

AD \_\_\_\_\_

GRANT NUMBER DAMD17-96-1-6212

TITLE: Tumor Cell De-Adhesion By Aberrant, Single Subunit Integrins

PRINCIPAL INVESTIGATOR: Ian Jongewaard, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute  
La Jolla, CA 92037

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19971210 059

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 1997	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 96 - 30 Jun 97)		
4. TITLE AND SUBTITLE Tumor Cell De-Adhesion By Aberrant, Single Subunit Integrins		5. FUNDING NUMBERS DAMD17-96-1-6212		
6. AUTHOR(S) Ian Jongewaard, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  The Burnham Institute La Jolla, California 92037		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012		10. SPONSORING/MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE		
13. ABSTRACT (Maximum 200)  Three alternatively spliced forms of the integrin $\beta 5$ subunit have been identified. Each mRNA splice removes one or more exons from the $\beta 5$ message, but does not alter the reading frame. Consequently, each splice variant encodes a protein that is lacking domains in the amino terminal portion of the molecule, near the ligand binding site. Each variant of $\beta 5$ contains transmembrane and cytoplasmic domains that are identical to wild-type $\beta 5$ . Analysis of the protein products of each splice variant by immunoprecipitation showed that each variant is translated to protein and expressed on the cell surface. However, the variants of $\beta 5$ do not dimerize with the $\alpha v$ subunit. This is the first identification of alternative splices in the ectodomain of an integrin that result in the display of a "single subunit integrin" on the cell surface.				
14. SUBJECT TERMS Breast Cancer integrins, metastasis cell adhesion			15. NUMBER OF PAGES 28	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

\_\_\_\_ Where copyrighted material is quoted, permission has been obtained to use such material.

\_\_\_\_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

X Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

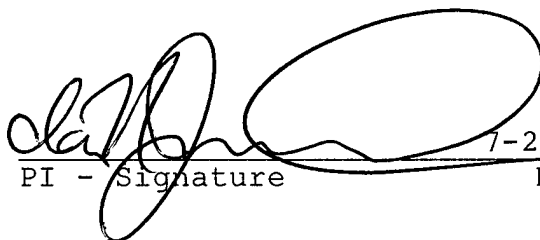
X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

\_\_\_\_ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

X In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

  
PI - Signature 7-25-97  
Date

## TABLE OF CONTENTS

	PAGE
FRONT COVER	1
REPORT DOCUMENTATION PAGE	2
FOREWORD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY OF REPORT	7-13
experimental methods and procedures	7
results and discussion	10
CONCLUSIONS	14
REFERENCES	15
APPENDIX	18-28
figures and figure legends	18

## INTRODUCTION

Integrins are non-covalently associated  $\alpha\beta$  heterodimers that mediate cell adhesion and cell migration. Integrins have key roles in thrombosis, atherogenesis, tumor dissemination, angiogenesis and viral infection (1-4). Integrins direct many cell-matrix contacts and also transmit vital cellular signals (5,6). The subject of this study is the integrin  $\beta 5$  subunit, which forms a heterodimer with  $\alpha v$  and can bind to the RGD tripeptide motif (7). The  $\alpha v\beta 5$  integrin is known to be a cell surface receptor for many adenoviruses (4,8) and to play a crucial role in angiogenesis (9). The  $\beta 5$  subunit is most closely related to the integrin  $\beta 3$  subunit (10,11). The two proteins are 56% identical at the amino acid level and the  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins share many of the same ligands (7,12).

The integrity of the integrin  $\alpha\beta$  heterodimer is thought to be essential for expression on the cell surface and for ligand binding function (13-16). Considerable evidence also demonstrates that the cytoplasmic tails of the integrin  $\alpha\beta$  heterodimer interact to control signal transduction (17-20). For example, deletions within either the cytoplasmic tail of the  $\alpha$  or  $\beta$  subunit can change the affinity of the integrin for ligand (20), suggesting that the interaction between the two tails places a conformational constraint on the receptors ectodomain. A more in-depth analysis has indicated that a salt bridge may physically connect the two cytoplasmic tails (21). When this salt bridge is disrupted, the affinity state and signaling properties of the integrin can be altered. Other data, however, suggests that the cytoplasmic tails of the integrin  $\alpha$  and  $\beta$  subunits may act autonomously. For example, yeast two-hybrid screens have identified proteins that will bind to the cytoplasmic tails of the integrin  $\beta$  subunits (22,23), and affinity chromatography has shown that calreticulin can bind to a conserved motif within the cytoplasmic tails of the  $\alpha$  subunits (24).

Other studies have shown that when  $\beta$  subunit tails are expressed as fusion proteins with unrelated membrane proteins, they can direct the localization of those proteins to focal adhesion sites (25). Similarly, fusion proteins containing an integrin cytoplasmic tail have been shown to negatively regulate cell adhesion (26) and to interfere with signaling (27). These studies have been taken as an indication that the cytoplasmic tail of the  $\beta$  subunit can function independently. Despite the evidence to indicate that the  $\alpha$  and  $\beta$  subunit cytoplasmic domains may have autonomous functions, no data have indicated how these domains could be displayed in the absence of their partner in a physiologic setting.

Here, evidence is presented challenging the doctrine that integrins exist solely as  $\alpha\beta$  heterodimers. The integrin  $\beta 5$  subunit is subject to a series of novel and highly specific alternative splices. Surprisingly, the splice variants are expressed on the cell surface as single-subunit integrins. Each splice variant contains a transmembrane domain and a cytoplasmic tail. Thus, the variants of  $\beta 5$  represent the first identification of an autonomously expressed integrin subunit. This finding adds another level of diversity to the integrin protein family; and also suggests a potential physiologic means of modulating cell adhesion, cellular signaling and integrin subunit composition.

## EXPERIMENTAL METHODS AND PROCEDURES

*cDNA cloning and DNA sequence analysis.* Total RNA was isolated from MDA-MB-435 cells using RNazol (28). RNA was converted to first strand cDNA using reverse transcriptase and then used as template in PCR amplification with oligonucleotide primers designed to amplify overlapping portions of the known  $\beta 5$  cDNA. The amplified DNA bands were analyzed by gel electrophoresis. Bands that were smaller than the predicted size for a given primer pair were cloned into the pBluescript II vector (Stratagene) by taking advantage of unique restriction sites that were engineered into each of the primers. The complete DNA sequence of all clones was verified by sequencing from both strands (29).

*PCR analysis of alternative splice expression.* Total RNA was isolated from MDA-MB-435 cells and human foreskin fibroblasts as described above. This RNA was converted to first strand cDNA using reverse transcriptase and then used for PCR amplification. PCR amplification of specific splice variants was accomplished by using a single reverse primer for all of the splice variants. This "universal" reverse primer was used in combination with a forward primer specific to each splice variant. The universal reverse primer for all amplifications of the  $\beta 5$  splice variants was: 5'-CCTGGTGCCCAGGTAGCCGGGGCTG; The forward primer for the  $\beta 5$  D/H splice variant was: 5'-TCCAGGCAGCCGTCTGCAAGAGTA-3'; The forward primer for the  $\beta 5$  E/H splice variant was: 5'-ACACTGCATCCAACCAGATGAGTA-3'; The forward primer for the  $\beta 5$  G/I splice variant was: 5'-TGATTATTAATGCATACAATGCAT-3'; The universal reverse primer for all amplifications of the  $\beta 3$  sequence was: 5'-GACGGGCTGACCCTCTCGGGGGCTG-3'; The forward primer used in an

attempt to detect a  $\beta 3$  D/H splice variant was: 5'-TGCAGGCTACAGTCTGTGATAAAA-3'; The forward primer used in an attempt to detect a  $\beta 3$  E/H splice variant was: 5'-ACTCTGCCTCCACTACCATGAAAA-3'; The forward primer used in an attempt to detect a  $\beta 3$  G/I splice variant was: 5'-TCATTGTTGATGCTTATGGGGTGA-3'). Amplified DNA fragments were visualized using agarose gel electrophoresis and ethidium bromide staining.

*Subcloning and transfection of 293 cells with the splice variants.* The overlapping fragments of  $\beta 5$  cDNA generated while cloning the portions of the cDNA that are alternatively spliced, were pieced together to produce full length cDNA and were used as a backbone for subcloning in the alternatively spliced exons. Each of these cDNA were then subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Human embryonic kidney 293 cells were transfected with each of the splice variants using cationic lipid transfer (DOTAP, Boehringer Mannheim) as outlined in the literature accompanying the product. Following selection with G418 (200ug/ml, Life Technologies), stable cell lines were used for the surface expression and subunit association experiments.

*Cell Surface iodination and immunoprecipitation.* The immunoprecipitations of  $^{125}\text{I}$ -labeled cells were carried out essentially as described (30). Protein bands were visualized by SDS PAGE on 7.5% acrylamide gels followed by autoradiography. The amount of each subunit remaining after immunodepletion was quantified from the intensity of the immunoprecipitated bands using a Bio Rad GS 525 Molecular Imager System.



*Antibody production.* Polyclonal antiserum was raised against an 11 amino acid peptide which corresponds to the splice junction at the boundary of exons D and H of  $\beta 5$  (protein sequence = gln-ala-ala-val-cys-lys-ser-ile-arg-ser-lys). Antisera was raised by conjugating the peptide to Keyhole Limpet Hemocyanin and inoculating rabbits with the conjugated antigen as described(31). Attempts to obtain polyclonal antisera against the other splice variants of  $\beta 5$  have been unsuccessful to date.

## RESULTS AND DISCUSSION

### *Identification of Alternatively Spliced forms of the Integrin $\beta 5$ Subunit in Breast Carcinoma Cells*

This study was initiated after it was observed that immunoprecipitation of the integrin  $\beta 5$  subunit from MDA-MB-435 breast carcinoma cells yielded multiple protein bands. Both a monoclonal antibody and a polyclonal antiserum raised against the  $\beta 5$  subunit immunoprecipitated a broad protein band with a mass substantially lower than wild-type  $\beta 5$  (Figure 1). This additional band represents a significant portion of the protein immunoprecipitated with antibodies against  $\beta 5$  (~30%). We hypothesized that this broad band is related to  $\beta 5$ . A reverse transcriptase polymerase chain reaction (rtPCR) cloning strategy was used to amplify cDNA's from the MDA-MB-435 cells which encode the  $\beta 5$ -like protein. Three PCR products of aberrant size were amplified and cloned. Each clone is an alternatively spliced form of  $\beta 5$  (Figure 2). All three alternatively spliced forms of  $\beta 5$  are lacking exons in the amino-terminal one third of the protein. The splice variants of  $\beta 5$  are designated D/H, E/H and G/I, to connote the exons which are contiguous within the splice. The D/H variant, for example, is lacking exons E, F and G placing the D and H exons in juxtaposition.

### *The mRNA Encoding the Integrin $\beta 5$ Subunit is Alternatively Spliced but mRNA Encoding $\beta 3$ is not*

A PCR strategy was used to determine whether the alternative splices of  $\beta 5$  are expressed in a non-transformed cell line and to determine whether the closely related integrin  $\beta 3$  subunit is similarly spliced. Each splice variant of  $\beta 5$  is present in both MDA-MB-435 cells and in normal human foreskin fibroblasts (Figure 3) indicating that cellular transformation is not necessary for the

splicing of this mRNA. The integrin  $\beta 3$  subunit, which is also expressed by both cell types, is not spliced (Figure 3). Consequently, the novel splicing mechanism can distinguish the mRNA's encoding  $\beta 3$  and  $\beta 5$ , even though they are highly related (11). Additional PCR reactions proved that the mRNA encoding each splice variant is mature (polyadenylated) and is also contiguous with the remainder of the wild-type  $\beta 5$  sequence (not shown).

*Variant Forms of the  $\beta 5$  Subunit are Expressed on the Cell Surface as Single Subunit Integrins*

Each spliced form of  $\beta 5$  is translated to protein and expressed on the cell surface. Human embryonic kidney 293 cells, which express only low levels of endogenous  $\beta 5$ , were transfected with cDNA's encoding each of the splice variants. The transfected 293 cells expressed the alternatively spliced forms of  $\beta 5$  on the cell surface (Fig. 4A, arrows). The  $\alpha v\beta 5$  heterodimer that is endogenously expressed by the 293 cells co-immunoprecipitates in these experiments. Each spliced version of  $\beta 5$  is lacking a domain of the  $\beta$ -subunit thought to be essential for  $\alpha\beta$  association (15,32,33); therefore, we examined whether the splice variants associate with  $\alpha v$ . To distinguish the expression of the new proteins as autonomous  $\beta$ -subunits from their potential association with  $\alpha v$ , lysates from the transfected 293 cells were immunodepleted of  $\alpha v$ -associated protein. Subsequently, the lysate was immunoprecipitated with antibodies against  $\beta 5$ . Immunodepletion of the  $\alpha v$  integrin did not reduce the level of any of the alternatively spliced forms of  $\beta 5$  on the cell surface (Fig. 4B). This immune-depletion did, however, deplete the endogenous  $\beta 5$ . Therefore, unlike  $\beta 5$ , which pairs with  $\alpha v$ , each variant of  $\beta 5$  is expressed on the cell surface as a single subunit integrin. To independently confirm that the splice variants are expressed autonomously, immunoprecipitations were performed

with an antibody raised against a synthetic peptide corresponding to the protein sequence at the D/H splice junction. As shown in Fig. 4C, this polyclonal antibody immunoprecipitated a single band from the MDA-MB-435 cells, with a mass corresponding to the predicted mass of the D/H variant. Thus, the D/H splice variant of  $\beta 5$  is expressed endogenously by the MDA-MB-435 line and is displayed on the cell surface without associating with  $\alpha v$ . Attempts to raise antibodies against the other splice junctions have been unsuccessful.

Prior to this report it was believed that all integrins existed as non-covalently associated  $\alpha\beta$  heterodimers. However, the data presented here demonstrate the existence of three splice variants of the integrin  $\beta 5$  subunit that do not associate with any  $\alpha$ -subunit partner. Each splice eliminates a segment of the ectodomain of  $\beta 5$  while maintaining the protein reading frame. Therefore, all three of the splice variants have a modified ectodomain, but intact transmembrane domains and cytoplasmic tails. Thus, the mRNA splicing enables the unique display of the  $\beta 5$  cytoplasmic tail in a form that is uncoupled from a subunit association and also from normal ligand binding function.

The function of the spliced version of  $\beta 5$  are not immediately evident, but prior study suggests that they could modulate integrin-dependent signaling. Several reports indicate that a disruption in the integrity of the integrin  $\alpha\beta$  heterodimer, particularly any interactions within the cytoplasmic tails, could regulate both "inside out" and "outside-in" signal transduction. For example, the autonomous expression of a cytoplasmic domain from an integrin  $\beta$  subunit is known to regulate integrin mediated signaling (27). When expressed as a fusion protein with the IL-2 receptor, the cytoplasmic domain of integrin  $\beta 3$  subunit is sufficient to stimulate integrin-specific tyrosine phosphorylation (19). Similarly, autonomously expressed  $\beta$ -subunit cytoplasmic tails can act as

trans-dominant inhibitors of integrin function because of their ability to induce cellular de-adhesion (26) and inhibit "inside-out" signal transduction (27). The data presented here, showing that alternative splices of  $\beta 5$  are displayed as single subunit integrins, suggest that alternative splicing of the message encoding  $\beta 5$  may be a physiologic means of displaying an autonomous integrin  $\beta$ -subunit positioned at the cell surface.

## CONCLUSIONS

Three novel alternative splice forms of the  $\beta 5$  integrin subunit have been identified in human breast carcinoma cells. These alternative splice forms appear to be expressed on the cell surface as single subunits. The next phase of this project is to test the hypothesis that the alternative splice variants can modulate adhesion and migration of tumor cells. Additionally, the abundance and localization of the alternative splice forms will be determined in tumors and tumor derived cell lines. Potential correlation with tumor progression and metastatic potential will be assessed to determine if these splice variants are useful as tumor markers.

## REFERENCES

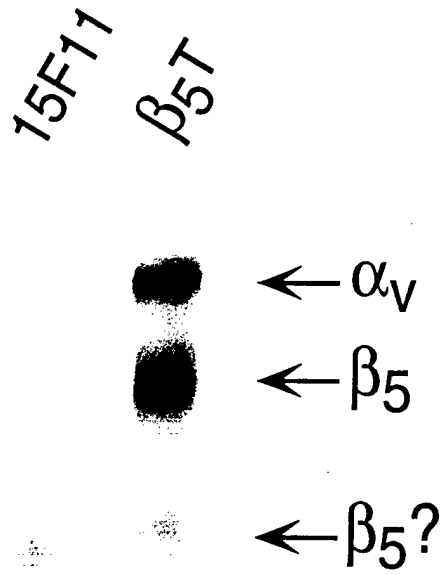
1. Ruoslahti, E. and Giancotti, F. G. (1989) *Cancer Cells* **1**, 119-126
2. Hynes, R. O. (1987) *Cell* **48**, 549-550
3. Albelda, S. M. and Buck, C. A. (1990) *FASEB J.* **4**, 2868-2880
4. Mathias, P., Wickham, T., Moore, M., and Nemerow, G. (1994) *J. Virol.* **68**, 6811-6814
5. Hynes, R. O. (1992) *Cell* **69**, 11-25
6. Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8392-8396
7. Smith, J. W., Vestal, D. J., Irwin, S. V., Burke, T. A., and Cheresch, D. A. (1990) *J. Biol. Chem.* **265**(19), 11008-11013
8. Wickham, T. J., Filardo, E. J., Cheresch, D. A., and Nemerow, G. R. (1994) *J. Cell Biol.* **127**, 257-264
9. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresch, D. A. (1995) *Science* **270**, 1500-1502
10. McLean, J. W., Vestal, D. J., Cheresch, D. A., and Bodary, S. C. (1990) *J. Biol. Chem.* **265**, 17126-17131
11. Ramaswamy, H. and Hemler, M. E. (1990) *EMBO J.* **9**, 1561-1568
12. Hu, D. D., Lin, E. C. K., Kovach, N. L., Hoyer, J. R., and Smith, J. W. (1995) *J. Biol. Chem.* **270**, 26232-26238
13. Phillips, D. R., Charo, I. F., Parise, L. V., and Fitzgerald, L. A. (1988) *Blood* **71**, 831-843
14. Cheresch, D. A. and Harper, J. R. (1987) *J. Biol. Chem.* **262**, 1434-1437
15. Nelson, C., Rabb, H., and Arnaout, M. A. (1992) *J. Biol. Chem.* **267**, 3351-3357
16. O'Toole, T. E., Loftus, J. C., Plow, E. F., Glass, A. A., Harper, J. R., and Ginsberg, M. H. (1989) *Blood* **74**, 14-18
17. Yamada, K. M. and Miyamoto, S. (1995) *Curr. Opin. Cell Biol.* **7**, 681-689

18. Rosales, C., O'Brien, V., Kornberg, L., and Juliano, R. (1995) *Biochim. Biophys. Acta* **1242**, 77-98
19. Akiyama, S. K., Yamada, S. S., Yamada, K. M., and LaFlamme, S. E. (1994) *J. Biol. Chem.* **269**, 15961-15964
20. O'Toole, T. E., Katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J., and Ginsberg, M. H. (1994) *J. Cell Biol.* **124**, 1047-1059
21. Hughes, P. E., Diaz-Gonzalez, F., Leong, L., Wu, C., McDonald, J. A., Shattil, S. J., and Ginsberg, M. H. (1996) *J. Biol. Chem.* **271**, 6571-6574
22. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996) *Nature* **379**, 91-96
23. Shattil, S. J., O'Toole, T., Eigenthaler, M., Thon, V., Williams, M., Babior, B. M., and Ginsberg, M. H. (1995) *J. Cell Biol.* **131**, 807-816
24. Rojiani, M. V., Finlay, B. B., Gray, V., and Dedhar, S. (1991) *Biochemistry* **30**, 9859-9866
25. LaFlamme, S. E., Thomas, L. A., Yamada, S. S., and Yamada, K. M. (1994) *J. Cell Biol.* **126**, 1287-1298
26. Lukashev, M. E., Sheppard, D., and Pytela, R. (1994) *J. Biol. Chem.* **269**, 18311-18314
27. Chen, Y. P., O'Toole, T. E., Shipley, T., Forsyth, J., LaFlamme, S. E., Yamada, K. M., Shattil, S. J., and Ginsberg, M. H. (1994) *J. Biol. Chem.* **269**, 18307-18310
28. Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156-159
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
30. Cheresch, D. A. and Spiro, R. C. (1987) *J. Biol. Chem.* **262**, 17703-17711
31. Harlow, E. and Lane, D. (1988) *Antibodies. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Kishimoto, T. K., O'Conner, K., and Springer, T. A. (1989) *J. Biol. Chem.* **264**, 3588-3595



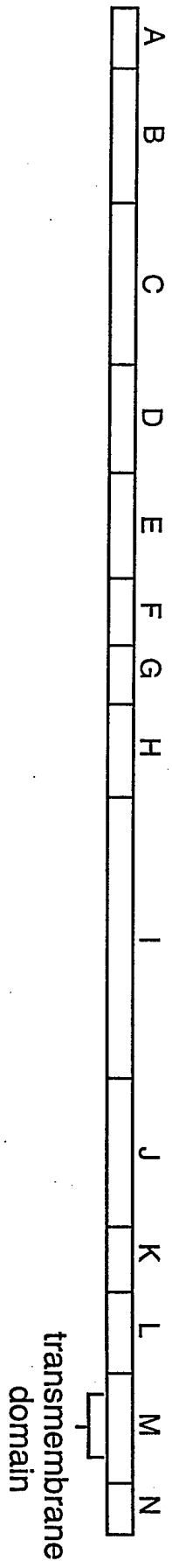
33. Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., and Yamada, K. M. (1988) *EMBO J.* **7**, 4093-4099
34. Stuiver, I. S. and Smith, J. W. (1995) *Hybridoma* **14(6)**, 545-550
35. Sago, K., Ross, F. P., Martin, J., Li, C-F., Chappel, J., Mimura, H., Reichardt, L. F., Venstrom, K., Teitelbaum, S. L., and Cao, X. (1993) *J. Bone Miner. Res.* **8**, S121
36. Lanza, F., Kieffer, N., Phillips, D. R., and Fitzgerald, L. A. (1990) *J. Biol. Chem.* **265**, 18098-18103
37. Real, F. X., Houghton, A. N., Albino, A. P., Cordon-Cardo, C., Melamed, M. R., Oetten, H. F., and Old, L. J. (1985) *Cancer Res.* **45**, 4401-4411

**Figure 1**     **Immunoprecipitation of multiple forms of  $\beta 5$  from MDA-MB-435 cells.** The integrin  $\beta 5$  subunit was immunoprecipitated from MDA-MB-435 breast carcinoma cells using the monoclonal antibody 15F11, which recognizes the  $\beta 5$  subunit (34), and with a polyclonal antibody raised against the cytoplasmic tail of  $\beta 5$ , designated as  $\beta 5T$  (35). Arrows indicate the positions of  $\alpha v$ ,  $\beta 5$ , and a protein of lower mass which we hypothesized was related to  $\beta 5$ . This experiment was repeated at least three times, each yielding the same result.

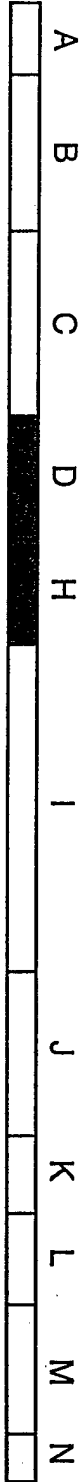


**Figure 2. Exon Map of the Alternatively Spliced Forms of  $\beta 5$ .** PCR cloning was used to identify three novel mRNA's encoding  $\beta 5$ -related sequences from the MDA-MB-435 cells. The nucleotide sequences determined by dideoxy sequencing showed that each clone contained a discontinuous segment of  $\beta 5$ . A comparison of each clone with the known genomic structure of both the integrin  $\beta 1$  and  $\beta 3$  subunits (36) revealed that the novel cDNA's encoded alternatively spliced forms of  $\beta 5$ . Each clone has exon junctions identical to those reported from the genomic sequences of  $\beta 1$  and  $\beta 3$ . The deduced exon structure of each new form of  $\beta 5$  is shown.

# Full Length Beta 5



D/H



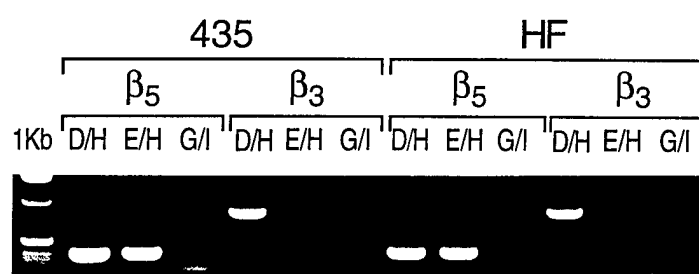
E/H



G/I



**Figure 3. The  $\beta 5$  Splicing Mechanism Bypasses the mRNA Encoding  $\beta 3$ .** A reverse transcriptase PCR amplification strategy was established to detect the mRNA encoding the  $\beta 5$  splice variants and to determine whether the closely related  $\beta 3$  subunit is similarly spliced. These amplification reactions were designed to distinguish the spliced mRNA from wild-type mRNA. Primers for the  $\beta 5$  splices were designed to hybridize across the 5' end of each splice junction, and to amplify only the splice variants. Similar primers were used to identify analogous splicing within the  $\beta 3$  message, although the primers used to amplify a D/H splice variant of  $\beta 3$  were also designed to amplify a segment of the full-length  $\beta 3$  (732 base pairs) as an internal control. mRNA samples from the both MDA-MB-435 breast carcinoma cells and from normal human foreskin fibroblasts were analyzed. The predicted sizes of the amplified fragments are; 384 base pairs for the D/H and E/H splice variants and 249 base pairs for amplification of the G/I splice variant. All of the PCR analysis was repeated at least three times with equivalent results.

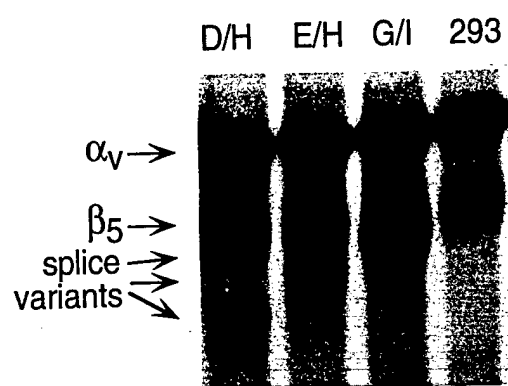


**Figure 4. The Variant Forms of  $\beta 5$  are Expressed on the Cell Surface as Single-subunit Integrins.** (A), The ability of each splice variant of  $\beta 5$  to be expressed as protein was determined by transfecting kidney 293 cells with expression vectors containing the cDNA encoding each splice variant. The 293 cells were chosen because they express the  $\alpha v$  subunit, but only low levels of endogenous  $\beta 5$ . The cell lines expressing each variant were surface labeled with  $^{125}\text{I}$ Na and then immunoprecipitated as described (30). Arrows indicate the position of each splice variant and endogenous  $\beta 5$  on the polyacrylamide gel. (B), To determine whether the spliced versions of  $\beta 5$  associate with  $\alpha v$ , the  $\alpha v$  subunit was immunodepleted from surface labeled protein samples prior to immunoprecipitation. The  $\alpha v$  specific monoclonal antibody L230 (37) was used to immunodeplete cell lysates from the three transfected cell lines. Immunodepletion was followed by immunoprecipitation with 15F11 to precipitate any remaining wild-type  $\beta 5$  or  $\beta 5$  splice variant. The cell lines from which the lysates were prepared are listed above the graph and the individual subunits from each of the cell lines are listed below. Each depletion was repeated at least three times with equivalent results. (C), The D/H splice variant can be immunoprecipitated as a single subunit from MDA-MB-435 cells. Antisera raised against the D/H peptide was used to immunoprecipitate

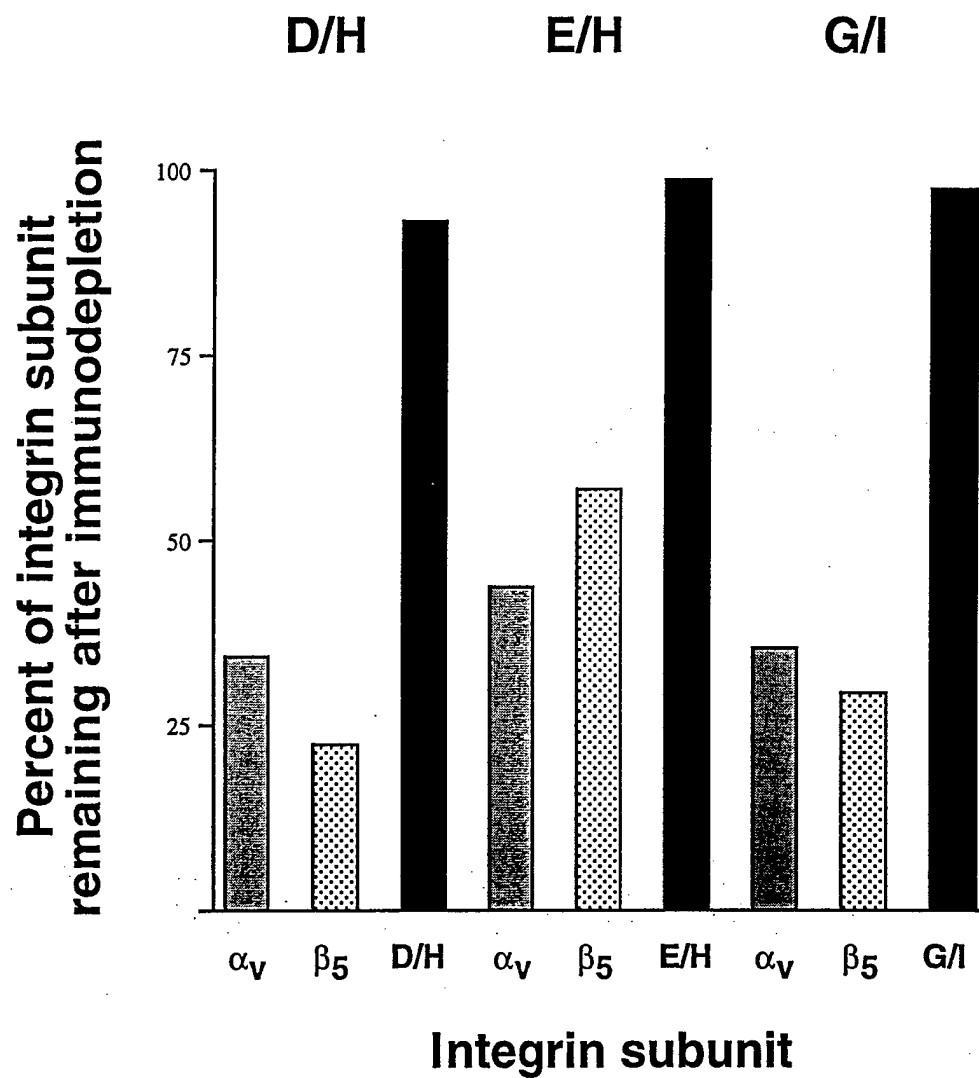


lysates from radiolabeled MDA-MB-435 cells. lane 1, immunoprecipitation with the anti-peptide antibody; lane 2, immunoprecipitation with the anti-peptide antibody in the presence of 10 mg/ml D/H peptide; lane 3, immunoprecipitation with the anti-peptide antibody in the presence of 10 mg/ml unrelated peptide (with sequence ser-pro-ala-ser-ser-phe-his-val-leu-arg-ser-leu). This experiment was repeated twice with the same results.

**A**



**B**



C

